Towards Improved Biomarker Studies of Cervical Neoplasia

Effects of Precolposcopic Procedures on Gene Expression Patterns

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Abstract: Among tumor sites, cervical cancer offers an ideal model for investigating differences in gene expression associated with transitions from normal to precancer and invasion to cancer. To evaluate the validity of assessing gene expression in cervical tissues acquired in a clinical setting, we investigated whether standard procedures, namely the application of acetic acid and/or Lugol's iodine, employed for the visualization of colposcopically directed biopsies, altered patterns in oligonucleotide (oligo) arrays. We compared microarray profiles from six women, each with three adjacent tissue samples removed from benign hysterectomy specimens and treated as follows: immediately frozen, acetic acid application only, acetic acid, and Lugol's iodine. Of the 22,464 original spots on the microarray, 4,850 spots were expressed at detectable levels for further evaluation upon data normalization and filtration. For each spot, the difference between topical applications was computed, and P values were calculated using a bivariate T^2 test. Upon adjustment for multiple comparisons using both the Holm's and Hochberg's procedures as well as the False Discovery Rate (Benjamini-Hochberg and Benjamini-Yeuketili [BY]), we failed to identify genes differentially expressed and conclude that standard precolposcopic procedures do not substantially affect the overall gene expression patterns in the normal cervix.

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Anonymized samples were obtained, in accordance with US Department of Health and Human Services guidelines. The institutional review boards at the NIH and at the University of Oklahoma approved this study.

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DNA microarrays allow the estimation of expression levels for thousands of genes within a selected sample. Expression profiles have enormous potential to improve disease classification and subclassification[1] and predict clinical diagnoses and survival[2–5] and response to treatment.[5] However, achieving accurate analyses of gene expression is complex, requiring meticulous quality assurance for the entire process, from the collection of biologic samples in the clinical setting to tissue preservation and processing and extending through data analysis and interpretation. The importance of validated quality assurance measures for tissue acquisition is receiving increasing attention.[6–8]

To date, the effects of clinical procedures related to specimen collection on gene expression have received limited attention, although these effects are highly relevant to the translation of biomarker discovery into clinical practice. This report examines whether routine procedures for performing colposcopically directed biopsies affect gene expression in cervical tissues. Briefly, cervical tissue acquisition procedures (eg, biopsies or Loop Electrosurgical Excision Procedures [LEEPs]) during a colposcopic visit include visualization of the cervix after application of 3% to 5% acetic acid and/or iodine to delineate potential lesions.[9] Cervical cancer precursor lesions turn white after application of acetic acid and fail to stain purple with iodine. These routine aspects of clinical practice could alter the detectable gene expression patterns in the tissue biopsies. Morphologic artifacts attributed to the iodine in Lugol's solution have been described in formalin-fixed, paraffin-embedded cervical tissue sections.[10] To assess this concern, we assessed the effects of acetic acid and Lugol's iodine on RNA quality, quantity, and resulting microarray profiles of normal cervical tissue, under tightly controlled conditions.

MATERIALS AND METHODS

Cervical Tissues and Processing Methods

Cervical biopsies were obtained from six women who underwent hysterectomy at the University of Oklahoma. Procedures were approved by the University of Oklahoma

Health Sciences Center and NCI Institutional Review Boards. Acetic acid (5%) followed by Lugol's iodine (5% Iodine, 10% Potassium Iodide; Premier Medical Supplies, Plymouth Meeting, PA) was applied to cervical tissues prior to hysterectomy. Each application was applied for 3 minutes until biopsy. For each woman, three adjacent tissue biopsies were taken: (1) tissue with neither acetic acid nor Lugol's iodine, (2) tissue with acetic acid application only, and (3) tissue with acetic acid and Lugol's iodine. Tissues were immediately placed in cryovials and snap-frozen in liquid nitrogen in a Dewar Flask (within 2 minutes) in the clinic until storage at −80°C prior to processing.

RNA Preparation and Extraction

Tissue weight was obtained by weighing while frozen. The tissue was then transferred to a tube containing at least 12 volumes (minimum volume of 1 mL) of RNAaqueous Lysis Buffer (Ambion) and immediately homogenized with a Polytron Homogenizer (Brinkmann) using the PTA7 probe. Tissue debris was removed by centrifugation at 14k rpm in a microfuge tube. Total cell RNA was then prepared according to the RNAqueous instructions and without the optional LiCl precipitation step. RNA concentration was determined using the RiboGreen assay (Molecular Probes) and quality using the RNA 6000 nano assay on the Bioanalyzer 2100 (Agilent Technologies). Contaminating DNA was removed using DNA-*free* DNAse treatment and Removal Reagent (Ambion) following manufacturer's instructions.

Evaluation of RNA Quality and Quantity

Extracted RNA 28s:18s ratios were calculated for each specimen by application; RNA integrity was thus evaluated by comparing the 28s:18s ribosomal RNA (rRNA) signal ratio. The optimal ratio was 2:1, and values greater than 1.5 were accepted as an indication of no RNA degradation.

Microarray Analysis

RNA Amplification/aRNA Preparation

Labeled, amplified antisense RNA (aRNA) was prepared using the amino allyl MessageAmp kit (Ambion) starting with 1 μg total RNA and following the manufacturer's protocol except as noted below. The cDNA synthesis reaction creates double stranded cDNA with a T7 promoter at the 3′ end. This cDNA was used as template for an in vitro transcription (IVT) reaction using T7 polymerase, generating labeled aRNA. IVT was carried out for 6 hours at 37°C using a 3:1 ratio of aaUTP to UTP and a total UTP concentration of 7.5 mmol/L. The cDNA template was removed from the IVT reaction by DNAse treatment prior to aRNA purification. aRNA was quantitated using a ND-1000 UV-Vis Spectrophotometer (Nanodrop Technologies) and then run on the Bioanalyzer RNA LabChip using the mRNA Smear Assay to determine the aRNA size profile.

aRNA Cy-3 and Cy-5 Labeling

We used a balanced design to eliminate dye-incorporation bias. The samples from half the individuals were labeled with Cy-3 while samples from the remaining individuals were labeled with Cy-5. Amino allyl-labeled aRNA was coupled with Cy-3 and Cy-5 NHS ester dyes at 25°C for 30 minutes using Cy Dye Post-labeling Reactive Dye Packs (Amersham Pharmacia). After quenching with hydroxylamine, the labeled aRNA was purified on filter cartridges and then analyzed on the Nanodrop spectrophotometer for RNA concentration and incorporation of Cy dyes. The frequency of incorporation (number of labeled nucleotides per 1000) was calculated using the formula foi = pmoles of dye incorporated × 320.5/ng of aRNA and ranged from 25 to 46.

Hybridization

One Cy-3 and 1 Cy-5 labeled aRNA (2 μg each) were combined and fragmented using the Ambion RNA Fragmentation Reagents following the manufacturer's protocol except that incubation was at 50°C for 15 minutes. The average size after fragmentation was 200 to 300 nt as determined on the Bioanalyzer. The labeled and fragmented aRNA was mixed with 10 μg human COT-1 DNA (Invitrogen) and 10 μg poly(dA)$_{40\text{-}60}$ (Amersham Biosciences) in water for a final volume of 21 μL, denatured at 100°C for 1 minute, and snap cooled on ice. Then an equal volume of 2× hybridization mix was added to give a final concentration of 25% formamide, 5× SSC, and 0.1% SDS. A pooled aRNA sample from a single woman with neither application of acetic acid nor Lugol's iodine was used as a common reference sample for all microarrays. We used a balanced design in which half of the arrays were hybridized with Cy-3–labeled reference aRNA while the remaining were hybridized with Cy-5–labeled reference aRNA to eliminate dye-incorporation bias. Technical reproducibility was evaluated with a reference self-self comparison.

Oligonucleotide microarrays were printed with 22,272 long (~70 nt) oligonucleotides in the NCI/CCR Microarray Center using the Operon (Qiagen) Human Genome Oligo Set version 2. Arrays were first prehybridized under Lifter Slips (Erie Scientific Company) with 5× SSC, 0.1% SDS, 1% BSA at 42°C for >1 hour, then washed sequentially for 2 minutes in distilled water and 100% isopropanol, and spin dried. The arrays were hybridized with aRNA in hybridization mix in GeneMachine HybChambers at 42°C for 24 hours and washed in 2× SSC, 0.1% SDS for 2 minutes, 1× SSC for 2 minutes, and 0.2× SSC twice for 1 minute each. Arrays were then dried by spinning in a slide rack in a Sorvall RT7 centrifuge at 700 rpm for 3 minutes. Arrays were scanned in a GenePix 4000B scanner (Axon Instruments) using GenePix Pro software.

Statistical Analysis

To assess whether cervical application of acetic acid or Lugol's iodine during colposcopy is likely to affect gene expression profile in collected tissue, we compared profiles from each of the 6 patients under the following three conditions: (1) application of neither acetic acid nor Lugol's iodine (none), (2) application of acetic acid only (AA), and (3) application of acetic acid followed by Lugol's iodine (AA/Lugol's).

Filtering and Normalization

After initial image analysis and processing, data from each array was stored in the NCI database (μAdb, available at

http://nciarray.nci.nih.gov/). An initial filtering was performed using tools available at the μAdb site, so that spots with intensities less than 500 and signal-to-noise ratios less than one were filtered out, as were spots with unreadable data. Of the 22,464 spots per slide, 71% of the spots were filtered out using this schema. The remaining images were visually inspected and screened to identify the most informative slides (eg, with complete information), upon which we considered the following distribution of slides for analysis.

For individual #1, there were two slides per application that were informative and acceptable upon visual inspection and we thus used data from both slides; for individuals #2 through 6, there were single slides used for analysis, per application. All following statistical analyses were carried out using the R statistical program[11] and tools developed using the Bioconductor suite of analytic programs (http://www.bioconductor.org).

Gene expression extracted at each spot from each slide was measured as the difference M in the $\log_2$-expression levels in the two channels, ie,

$$M = \log_2(\text{Cy5 expression}) - \log_2(\text{Cy3 expression}) = \log_2\left(\frac{\text{Cy5 expression}}{\text{Cy3 expression}}\right)$$

Cy3 and Cy5 labeling were reversed for half the specimens; this was conducted to minimize potential bias from fluorescent labeling or dye incorporation. The expression level at each spot for each channel was calculated as the mean foreground intensity minus the median background intensity. We considered the MvA plot for each slide, which plots the difference in $\log_2$-expression against the average $\log_2$-expression for the 2 channels on a slide. We then normalized each slide by fitting a loess or locally weighted regression line to the MvA plot by print-tip group and then adjusting the data so that the fitted line would be horizontal.[12]

Differential Expression

Differences in gene expression were measured between the treatment groups (1) AA and none (reference sample) and (2) AA with Lugol's and none. For each spot, the difference in M between treatment groups was computed, resulting in 2 statistics for each spot for each individual:

$$\mathrm{d_{AA}} = \mathrm{M_{AA}} - \mathrm{M_{none}} \text{ and } \mathrm{d_{lugols}} = \mathrm{M_{lugols}} - \mathrm{M_{none}}$$

For each spot we computed a pair $d = (d_{\mathrm{AA}}, d_{\mathrm{lugols}})$; these pairs are independent between individuals. With a total of 7 paired values, we thus jointly tested to see whether either of the two differences is different from 0 using a bivariate T^2 test, which in this case is an extension of the paired t test.[13] This allowed simultaneous evaluation of gene expression differences between acetic acid and no application and between acetic acid with Lugol's iodine and no application. Under the null hypothesis, with n (up to 7) independent observations, it follows a $F(2,n\text{-}2)$ distribution.

For individual #1, there were two slides per application. We thus performed the analysis using each of the following two ways of defining the application differences and found no differences in our conclusions. We initially ignored the correlation between the slides, thus treating them as independent; we did this with the recognition that the standard error would be underestimated, thus increasing the probability of identifying differentially expressed genes. We also considered the correlation between the slides; in this setting, the standard error would be larger and a null result here would be consistent with a null result when assuming independence. We therefore performed bivariate T^2 test assuming seven independent pairs of differences (when ignoring correlation) and assuming six independent pairs of differences (when considering correlation).

Of the spots remaining on each slide after data filtration, we identified those with complete data and thus remaining on all slides postfiltration and normalization. A spot is considered to have complete data if we can compute d (where $d = (d_{\mathrm{AA}}, d_{\mathrm{lugols}})$), for all the individuals, and thus data points for AA, Lugol's, and none. We also included in our analyses those spots considered to have "minimal" data, defined as the ability to compute at least four pairs overall. Because filtration was uniformly conducted, using these definitions, we have 2,765 spots (12.3%) with complete data and 4,850 spots (or 21.6% of the spots) with at least minimal data that remained after filtration.

We performed the bivariate T^2 test for all the spots with complete and minimal data, and obtained P values. We graphically present this data with a *volcano plot*, which plots $\log_{10}$ (P value) against

$$\|\overline{d}\| = \sqrt{\overline{d}^2_{\mathrm{AA}} + \overline{d}^2_{\mathrm{lugols}}}$$

which is the length of the average T^2 d for each spot or the average log-fold change for the two comparisons.

A multiple comparisons adjustment of all P values was subsequently conducted for both 2,765 and the 4,850 tests to control for false positives. We used two classes of methods: controlling the family-wise error rate (FWER) using procedures like the Bonferroni correction and controlling the false discovery rate (FDR) using the Benjamini-Hochberg and Benjamini-Yekutieli methods.[14–16] The number of spots that satisfy, for each α, a family-wise error rate of at most α for the FWER methods, and a false-discovery rate of at most α for the FDR methods was calculated. We also employed a graphical method for visually comparing the observed gene expression differences to that of the null hypothesis. Under the null hypothesis, the P values follow a uniform distribution over the interval (0,1). We thus constructed a quantile plot of the observed P values against the theoretical quantiles of the U (0,1) distribution.

RESULTS

Initial comparisons of RNA quality were made, comparing 28s:18s ratios for each specimen, by application.

A representative conventional gel-like image of capillary electrophoresis is shown in Figure 1A with 28s and 18s ribosomal RNA (rRNA) signals. In all women for all applications, the 28s:18s rRNA signal ratio was greater than 1.5, indicating no RNA degradation and thus no difference in RNA quality by treatment, as further evidenced by the lack of smearing patterns. As shown in Figure 1B, median aRNA size ranged from 500 to 1000 base pairs; the aRNA sizes did not appear to differ by treatment. As oligos used in the arrays are located between 600 to 1,000 nucleotides from the poly-A tail, the aRNA size was thus adequate to provide signals for the majority of spots on the array.

In our initial bivariate T^2 test, we observed a number of spots that appear to be differentially expressed. This is graphically represented in our volcano plot (Fig 2), which includes data from all individuals with at least minimal data. As seen in Figure 2, while a majority of spots appear to possess

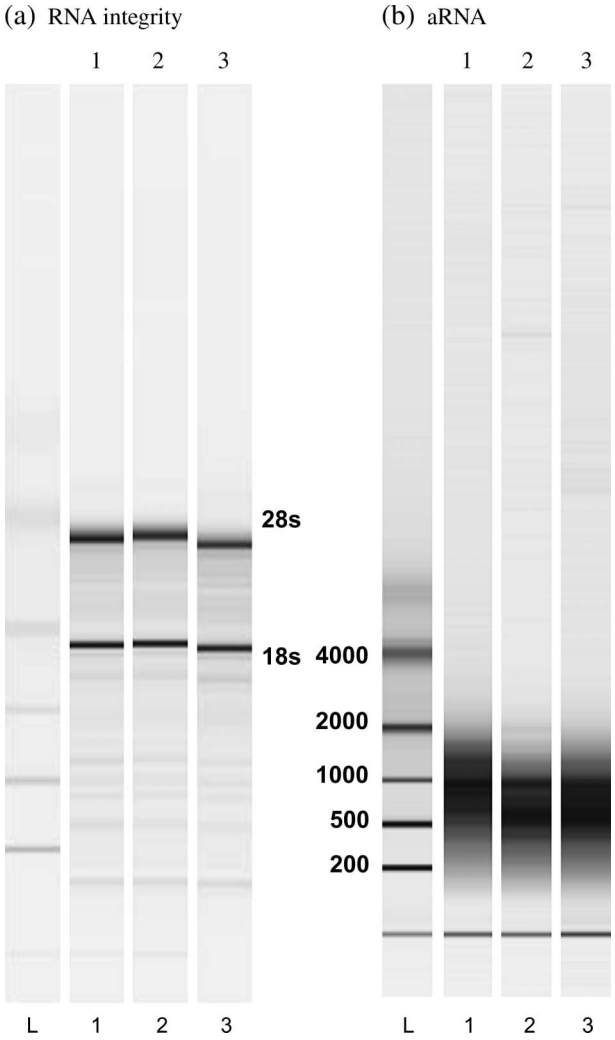

FIGURE 1. A, Agilent 2100 Bioanalyzer profiles of RNA extracted from cervix tissues by treatment: lane 1, no treatment; lane 2, acetic acid; and lane 3, acetic acid with Lugol's iodine. B, aRNA by treatment: lane 1, no treatment; lane 2, acetic acid; and lane 3, acetic acid with Lugol's iodine.

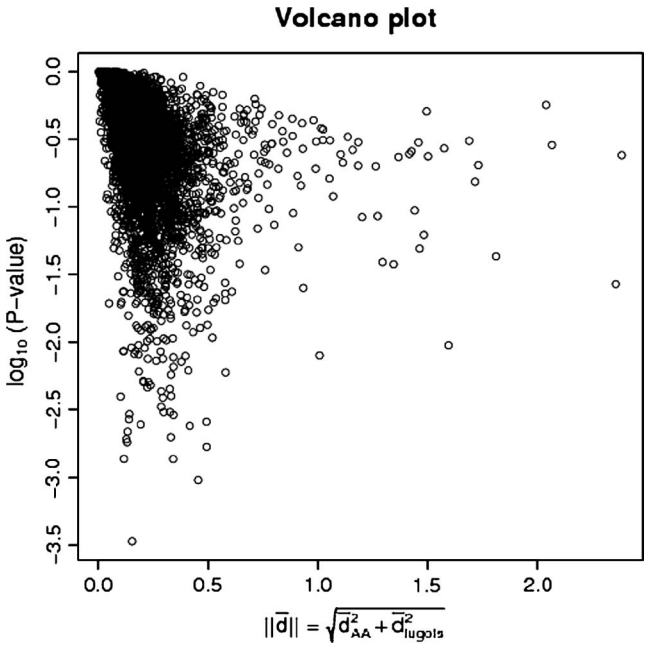

FIGURE 2. Volcano plot representing results from bivariate T^2 test, plotting P value (y-axis) by differential expression of treatments (x-axis), inlcuding spots with minimal data.

minimal differences in expression by treatment, there are evident spots with larger differences spanning across the length of the x-axis. Taking into consideration the P values on the y-axis, however, we observe that a number of spots with larger differences, such as those with up to a four-fold average difference between the treatment groups, are also those that do not correspond with a small P value. Likewise, those spots with low P values appear to have smaller differences in gene expression profiles. The analysis limited to the subset of 2,765 spots with complete data yielded similar results. In future analyses of disease, the minimal difference in gene expression accepted and the maximally acceptable P value will yield differential candidate genes for further investigation. Small P values for large differences in gene expression would, however, be optimal for biomarker discovery.

Table 1 displays the number of spots/genes that satisfy, for each level of significance (as determined by α), a family-wise error rate of at most α for the family-wise error rate (FWER) methods (Bonferroni, Holm, and Hochberg), and a false discovery rate of at most α for the False Discovery Rate (FDR) methods (BH and BY). Data are shown for analyses assuming seven independent samples, thus increasing the probability of identifying genes differentially expressed. Of the 275 spots initially identified with an alpha of 0.05, none of the spots were found to be significantly differentially expressed upon adjustment for multiple comparisons. In fact, at all levels of significance, by both Holm and Hochberg, no spots were found to be significantly differentially expressed. There were thus no genes identified in the FDR per BH and BY methods for further evaluation. Although a very small proportion of genes may be differentially expressed, these occur at extremely high alpha levels (eg, $\geq$0.85). A visualization of these data is shown in our quantile plot (Fig 3); as the figure

TABLE 1. Multiple Comparison Procedures and the Number of Spots Satisfying Each Criterion

		FWER Procedures			FDR Procedures	
α	*P* value	Bonferroni	Holm	Hochberg	BH	BY
0.05	275	0	0	0	0	0
0.1	587	0	0	0	0	0
0.15	879	0	0	0	0	0
0.2	1,183	0	0	0	0	0
0.25	1,507	0	0	0	0	0
0.3	1,782	0	0	0	0	0
0.35	2,065	0	0	0	0	0
0.4	2,350	0	0	0	0	0
0.45	2,619	0	0	0	0	0
0.5	2,880	0	0	0	0	0
0.55	3,086	0	0	0	0	0
0.6	3,324	0	0	0	0	0
0.65	3,553	0	0	0	0	0
0.7	3,767	0	0	0	0	0
0.75	3,947	0	0	0	0	0
0.8	4,140	0	0	0	0	0
0.85	4,327	0	0	0	2,966	0
0.9	4,492	0	0	0	3,731	0
0.95	4,680	0	0	0	4,311	0
1	4,850	4,850	4,850	4,850	4,850	4,850

shows, there is little deviation from the uniform distribution. Qualitatively similar results are obtained if we include spots present on at least 50% of the slides (5,780 spots).

Finally, we also conducted analysis of variance (ANOVA) for subject specific variability and found that for over 70% of the spots, the differences by subject accounted for over half the total variability, and in over 20% of the spots, it accounted for over 80% of the total variability (data not shown). This indicates that for most spots, the primary differences in expression are due to subject differences and not due to the application of acetic acid or Lugol's iodine.

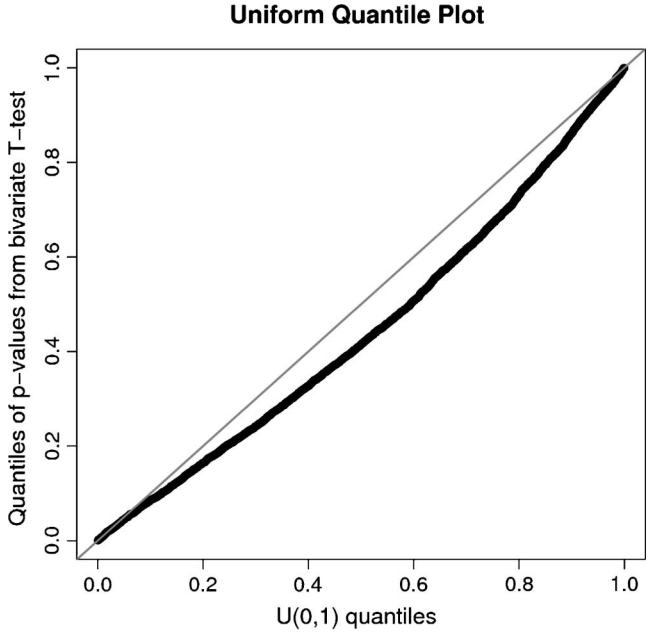

FIGURE 3. Quantile plot of observed *P* values against the uniform distribution.

DISCUSSION

Although advancing technology such as DNA microarrays now provide researchers with tremendous capabilities for interrogating gene expression patterns in the hopes of understanding carcinogenic processes,[17] adequate and uniform acquisition and processing of these tissues remains challenging. Numerous variables must be considered prior to the interpretation of downstream microarray results, including the disease state, the method by which the tissues were acquired, any applications applied prior to tissue acquisition, and the processing and subsequent preservation of the tissues. As has been suggested previously, gene expression profiles can change due to anoxia and ceasing circulation, making the time and temperature at which the samples are ex vivo critical.[18]

We conducted class comparisons between the different applications typically applied during colposcopic procedures prior to obtaining cervical samples. By calculating differences between gene expression patterns with the application of acetic acid compared with no application and Lugol's iodine with acetic acid compared with no application, we were able to calculate statistical significance of these gene expression profiles with an extension of a *t* test. Overall, we found no difference in gene expression due to the application of acetic acid or Lugol's iodine. Our use of the Holm and Hochberg procedures allowed us to control for multiple comparisons of the genes interrogated. Our additional analyses by the more liberal Benjamini-Hochberg (BH) assumes independence of tests, and Benjamini-Yeukatili (BY) allows dependence between tests, procedures that further allowed us to control for the false discovery rate; this method is more forgiving than the Holm and Hochberg control methods and retains more power for the tests. Adjusting for multiple comparisons by all methods, however, we show that at no level of significance do any of the genes possess differential expression by treatment.

In the current analysis, a reference design was used (as opposed to pairwise comparison) whereby we selected RNA from a single normal untreated tissue specimen where a large quantity was available to serve as the reference for all other samples. To ensure the highest quality RNA, all cervix tissues were snap frozen immediately upon biopsy in our attempts to reduce the effect of time on tissue acquisition. They were subsequently processed in an identical manner, thus allowing only the topical application to vary. For each of the six women with differential applications, multiple microarrays were conducted (as many as four) with a balanced design of reverse dye labeling to eliminate dye-incorporation bias and ensure highest quality of results.

Given that the hysterectomies were colposcopically normal, it is probably not surprising that a majority of spots were filtered out as we were not assessing expression differences expected with disease tissue. The preponderance of genes filtered out further support the notion that these

treatments are not affecting the overall expression patterns. Nevertheless, we cannot discount the possibility that transient or subtle but immeasurable differences may still have occurred. Acetic acid temporarily whitens abnormal cervix squamous epithelium, a result from dehydration and surface coagulation of cellular proteins.[19] Albeit temporary, it is plausible that such coagulation might result in differential gene expression patterns. Lugol's iodine stains brown upon its interaction with glycogen, which is found in normal, mature, squamous epithelium.[20] Because abnormal epithelium lacks intracytoplasmic glycogen, it is the nonuptake of iodine that marks lesional cervix tissue. Use of strong iodine solutions has been reported to result in histologic artifacts,[10] including cellular shrinkage and cytoplasmic vacuolization. Accordingly, our evaluation of the effects of iodine was important. As we are looking for large changes and artifacts in gene expression due to these applications, we are reassured that no notable alterations are identified.

In our study, we chose to preserve tissues and thus RNA quality and integrity via snap freezing in liquid nitrogen. We attempted to minimize hypoxic and stress-inducing events[6] by freezing the tissue immediately in the clinic without further handling or processing. Although we cannot entirely exclude the possibility that gene expression profiles might have changed from their in vivo status, we made every attempt to handle the specimens uniformly. Thus, any ex vivo effects related to delays in process or fluctuation in temperature were minimized. Nevertheless, a study limitation remains the comparison of 6 women and we cannot exclude potential differences in their tissue handling.

With the eventual goal of fully understanding cervical carcinogenesis, we are seeking to validate methods for DNA microarray analyses; this requires accurate analyses of gene expression entailing persistent and meticulous quality assurance during tissue collection, excision, preservation, and processing. Toward that goal, we thus believe this study demonstrates that standard colposcopic procedures of acetic acid and Lugol's iodine application in the cervix can be conducted without affecting the downstream gene expression profile, provided that all other aspects of tissue acquisition and processing are strictly controlled. To allow for such strict control of conditions, however, a limitation of the present study remains the use of nondiseased tissues.

Future efforts regarding clinical translation of any findings will likely require a number of meticulously conducted studies and cautious interpretations. To identify and validate biomarkers of risk for cervical cancer, identifying genes differentially expressed in normal, HPV-infected, precancer, and cancer tissues will be of enormous value. This will require microdissection of specific cell types; in our present analysis, although we made every attempt to biopsy predominantly the epithelial cells, it is possible that RNA from stromal cells might have diluted gene expression alterations in epithelial cells. As our goal remains the identification of a robust biomarker of which large changes in expression would be required, we believe our results remain valid as any dilutional effect would have likely inhibited only our ability to detect mild alterations.

Further studies should also include validation of differentially expressed genes prior to cancer development, requiring prospective data to assess their predictive value for cancer risk. The subsequent validation and application in accessible specimens (eg, cytology) will be critical for the large-scale application of any early detection biomarker in populations. Clinical translation will thus require careful validation of methods and vigilant quality control procedures in large-scale epidemiologic studies.

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